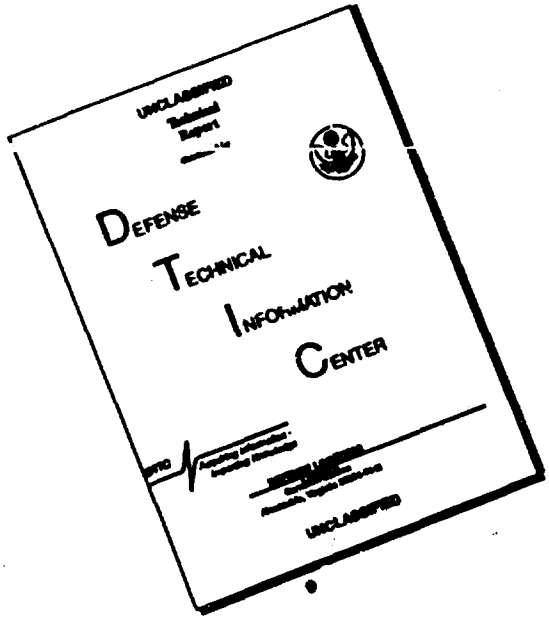
DISCLAIMER NOTICE



THIS DOCUMENT IS BEST QUALITY AVAILABLE. THE COPY FURNISHED TO DTIC CONTAINED A SIGNIFICANT NUMBER OF PAGES WHICH DO NOT REPRODUCE LEGIBLY.

Reprinted for private circulation from
THE JOURNAL OF INFECTIOUS DISEASES • VOL. 124, NO. 2 • AUGUST 1971

Evaluation of Resistance to Staphylococcal Enterotoxin B: Naturally Acquired Antibodies of Man and Monkey

Virginia G. McGann, J. Brent Rollins,* and Douglas W. Mason

From the United States Army Medical Research Institute of Infectious Diseases, Frederick, Maryland

Sera from residents of the United States and Southeast Asia were surveyed for the presence of antibody to highly purified staphylococcal enterotoxin B (SEB). Hemagglutination of ovine erythrocytes coupled to SEB was the method for detecting antibody, and immune-precipitation reaction in agar gel served as an indicator of IgG antibody activity. Approximately 70% of sera from the U.S. had HA antibody and 30% had precipitins, whereas all samples from Southeast Asia had HA activity and 90% had precipitating activity. Seroconversion occurred in individuals who had no known history of exposure to SEB. Experimental intoxication of rhesus monkeys indicated that repeated parenteral exposure to SEB was required to achieve the titers of antibody observed in human sera. Rechallenge by gavage was less effective, in that antibody stimulation occurred only in monkeys that were fed repeatedly with 100 median effective doses (ED_{50}). Resistance to a primary iv challenge with 1–25 ED_{50} was correlated with titers of naturally acquired HA antibody, but resistance to higher doses was related to presence of precipitins. Ten weeks after primary exposure, many monkeys lacking humoral antibody were resistant to a second iv challenge. In contrast, illness following primary challenge with 1–100 ED_{50} by gavage was unrelated to values of antibody, and the initial exposure had no affect on the response of seronegative monkeys to a second challenge by gavage.

Evidence that humoral antibody may be a factor in resistance against staphylococcal enterotoxemia has been derived primarily from demonstration of the efficacy of hyperimmune serum for prophylaxis and therapy or for neutralization of toxic activity [1–4]. There have been few attempts to evaluate host resistance by means of serologic techniques, and until highly purified preparations of toxin became available, interpretation of serologic findings presented serious difficulties.

Studies with hyperimmune sera from rabbits suggested that protective activity might be associated with precipitating antibody. In this regard, successful prophylaxis against iv challenge of monkeys was effected by several lapine type B antitoxins at dosages that contained equivalent quantities of antibody nitrogen, as determined by quantitative precipitin techniques [4]. With simian sera, however, protection did not appear to be related to the presence of precipitating antibody. Bergdoll [2] reported that some monkeys immunized against staphylococcal enterotoxin B (SEB) had precipitins but showed little resistance to this toxin, whereas other monkeys lacking precipitins were highly resistant. The serum dosage that conferred passive protection against oral SEB correlated roughly with resistance of the donor monkey. Little is known about incidence or significance of antibodies to enterotoxin in hu-

Received for publication February 10, 1971.

The authors thank Elizabeth O. Roberts, Eugene Massey, and Clarabelle Collins for excellent technical assistance.

In conducting research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences—National Research Council.

Please address requests for reprints to Dr. Virginia G. McGann, U.S. Army Medical Research Institute of Infectious Diseases, Frederick, Maryland 21701.

* Present address: College of Veterinary Medicine, University of California, Davis, California 95616.

8

man sera. In studies reported by Felsenfeld and Nasuniya [5], the complex antigenic composition of test materials precludes identification of specific antibody.

When highly purified preparations of SEB became available, it seemed appropriate to examine methods for detection of antitoxic activity, and to evaluate the use of antibody as a tool in epidemiologic survey, retrospective diagnosis, and estimation of resistance. This report describes such an investigation. Serologic procedures were restricted to those capable of detecting naturally acquired antibody in human sera. Surveys for incidence of antibody were conducted with groups of individuals from widely separated geographic areas, and the relationship of titer of antibody to resistance was evaluated by parenteral and alimentary challenge of rhesus monkeys (*Macaca mulatta*) with SEB.

Materials and Methods

Human sera were obtained from several sources. Preliminary investigations of serologic methods were made with 191 sera collected at random in a military dispensary in the eastern United States. Additional sera obtained between 1964 and 1969 from 515 healthy North American males, 18–24 years of age, during routine physical examination at the U.S. Army Medical Research Institute of Infectious Diseases were evaluated for the presence of antibody. For analogous studies of indigenous peoples of Southeast Asia, 413 sera from native villagers of South Viet Nam and Thailand were supplied by LtC. Harry G. Dangerfield, M.C., and Maj. Thomas J. Keefe, V.C., U.S. Army. All samples of serum were stored at −20 C.

Healthy rhesus monkeys weighing 2–3 kg were used for investigation of the relationship between naturally acquired antibody and resistance. Before experimental use, commercially obtained animals were conditioned for at least three months in optimal facilities for animal colonies. Enterotoxin was administered by injection into the femoral vein or by intubation of the stomach. Monkeys were under continuous observation for 12 hr after challenge and then observed twice daily for the next 10 days. Toxic response was defined as emesis and/or diarrhea within 12 hr, or death within 10 days. Samples of serum were obtained from all monkeys before the conditioning period, immediately before challenge, and at intervals for eight weeks after challenge.

A single lot of highly purified SEB, prepared and characterized by Schantz et al. [6], was used for titrations of antibody. The same preparation, or a partially purified lot containing 34% SEB, was used for challenge of monkeys. At equivalent concentrations of SEB, as determined by the Oudin technique [7], both preparations had the same toxic activity. The median effective dose (ED_{50}) for monkeys that had no antibody was 0.05–0.1 μg of SEB injected iv, or 4 μg of SEB administered by gavage (gi), per kg of body weight. Solutions for challenge were prepared in pyrogen-free physiologic saline, U.S.P. (Travenol Laboratories, Inc., Morton Grove, Ill.) at SEB concentrations of 0.05, 0.25, 1.25 and 10 μg/ml for iv challenge, and of 4, 40, and 400 μg/ml for gi challenge.

Sera were titrated for hemagglutinins, precipitins, and toxin-combining activity. Procedures described by Boyden [8] for preparation of sensitized, tanned erythrocytes (RBC) and by Gordon et al. [9] for RBC coupled to antigen with bis-diazotized benzidine (BDB) were used for study of hemagglutination reactions. Sensitization of tanned RBC was accomplished by treating a 2.5% suspension of tanned human RBC (type O) with five volumes of SEB diluted to 10 μg/ml in phosphate-buffered saline, *p*H 6.4; reactions of sensitized RBC with dilutions of inactivated serum were assayed in tube tests. Enterotoxin was coupled to ovine RBC at *p*H 7.3 in 0.15 M phosphate buffer by mixing a 50% RBC suspension with 10 volumes of SEB at 50 μg/ml, followed by addition of one volume of the optimal concentration of BDB, as determined by titration; sera for the tests were inactivated and absorbed with sheep RBC before assay in microtiter plates. Titers were recorded as the reciprocals of the highest dilution of serum that caused complete agglutination of treated RBC.

Precipitin reactions were studied in tubes, capillaries, and agar gel. The constant-serum method, using undiluted serum and variable amounts of SEB, was used for titrations in tubes and capillaries; tests were examined throughout a three-day interval for formation of rings or development of turbidity. Immunodiffusion reactions

were studied by constant-antigen and constant-serum methods in gel containing 1% Nobel agar (Difco), 0.038 M NaCl, and 0.062 M borate buffer, *p*H 8.3. In the constant-antigen procedure, 50-μl quantities of twofold dilutions of serum were observed for reaction with 0.25 and 1.0 μg SEB; in the constant-serum procedure, 50 and 100-μl quantities of undiluted serum were tested against 0.1–20 μg SEB. Reactions were recorded after three days at 30 C, and again after staining with a solution containing 0.01% trypan blue and 0.005% picric acid in 1% acetic acid. Titers were reported as the reciprocal of the highest dilution of serum that caused formation of a line of precipitate.

A modification of the technique of immunodiffusion in gel described by Wright [10] was used to evaluate toxin-combining activity of serum. Quantities of SEB, ranging from 0.01–2.0 μg, were incubated with 0.1 ml undiluted serum for 30 min at 37 C, and each mixture was tested for excess antigen and antibody in immunodiffusion plates. Uncombined antibody was detected by reaction between the test mixture and 0.25 μg reference SEB; uncombined SEB was detected by reaction between test mixture and reference antitoxin. If neither component was detected, the ratio of antigen to antibody was assumed to be at equivalence. The midpoint of the equivalence zone, converted to μg of SEB/ml of serum, was used as a measure of combining activity.

Determination of the class of immunoglobulin responsible for antibody activity was attempted. Immunoelectrophoresis, as described by Scheidegger [11], was performed on glass slides coated with 1% agarose (Kingman Optical Co., Washington, D.C.); protein patterns developed with lapine antiserum to whole serum from monkeys or antiserum to globulin from monkeys (Sylvana Co., Millburn, N.J.) were compared with the precipitin line developed with SEB. In addition, single factor, human antisera to IgG, IgA, and IgM (Hyland Laboratories, Los Angeles, Calif.) were used in immunodiffusion tests for identity with, or enhancement of, the SEB precipitin reaction. Representative sera were separated on columns of Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, N.J.), and fractions were tested for class of immunoglobulin with single factor, human antisera and for antibody activity with SEB.

Results

Comparison of serologic techniques. Hemagglutination of RBC coupled to SEB via BDB (BDB-HA) proved to be the most sensitive technique for detecting naturally acquired antibodies to SEB in human sera (table 1). BDB-HA titers ranged from 10 to 10,000, with a geometric mean of 180. Positive reactions with other serologic tests usually occurred in sera that had BDB-HA titers of >80. Some sera lacking hemagglutinins, however, had precipitating antibody and some with high BDB-HA titers showed no reaction by other methods of titration. Techniques of gel diffusion were required for demonstration of precipitin, and reactions of many sera were detected only after staining.

Human surveys. Incidence and titers of SEB antibody in sera from residents of the United States and Southeast Asia are shown in figure 1. Although the frequency distribution for the U.S. population represents combined data from groups of individuals tested over a period of five years, profiles of antibody were similar for each group and did not differ from that of the random specimens collected at the dispensary. Approximately 30% of the sera had no detectable antibody; 70% had hemagglutinins with a median titer of 40; and 25% had precipitins. Within this group,

Table 1. Comparison of serologic techniques for detection of naturally acquired antibodies to staphylococcal enterotoxin B (SEB) in human sera (191 subjects).

Titration	Positive sera	
	Percent	Geometric mean titer
Precipitin:		
Tube	0	0
Capillary	0	0
Immunodiffusion	28	2
Hemagglutinin*:		
Tanned RBC	34	34
BDB-RBC	75	180
Combining activity:		
μg SEB/ml serum	57	0.2

* Tanned human type O red blood cells sensitized with SEB; BDB-RBC = SEB coupled to ovine RBC with bisdiazotized benzidine.

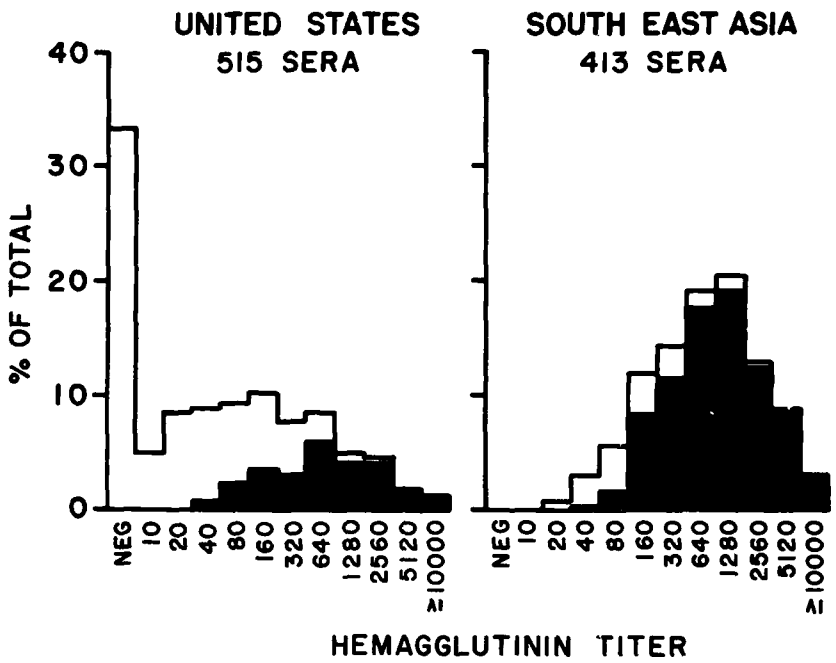


Figure 1. Frequency distribution of titers of HA antibody to enterotoxin B (clear areas) and precipitin reactions (dark areas) in sera from residents of the United States and Southeast Asia.

the incidence of precipitating antibody gradually increased with titer of HA antibody. When precipitins were present, antibody activity was predominantly associated with IgG immunoglobulins, but in their absence, activity might be found in any, or all, classes of immunoglobulin.

A second sample was obtained from 142 individuals 6–18 months after the first. No known experience with SEB occurred in the interim, but eight of 53 individuals who were originally antibody-negative developed hemagglutinins (range of titer, 40–320) and two developed precipitins (range of titer, 1–2). Essentially no change was observed in 38 individuals with low BDB-HA titers. Often, however, if the first serum had a high BDB-HA titer and no precipitating antibody, subsequent samples showed a loss in activity of HA antibody. In contrast, if both hemagglutinins and precipitins were present originally, BDB-HA titers remained constant but precipitins often decreased.

Tests with sera from 27 children, 6–14 years of age, suggested that an antigenic stimulus occurred during preschool years. The incidence of hemagglutinins and precipitins in sera from 6–7-year-old children was essentially the same as previously noted for samples from adult residents of the U.S.

Incidence and titer of SEB antibodies were significantly higher in sera from residents of Southeast Asia (figure 1). All sera had hemagglutinins with a median titer of 640; the distribution of BDB-HA titers followed a normal curve. Approximately 90% of the sera had precipitins; the incidence of precipitating antibody increased abruptly at HA titers $\geq$160.

Survey of simian sera. Monkeys were tested for SEB antibody after arrival at U.S. Army Medical Research Institute of Infectious Diseases. From 4%–40% of the monkeys in each shipment were found to have HA antibody. Overall mean incidence for 23 shipments was 16% (SD, 6%) and the median titer of positive monkeys was 80. The frequency distribution of precipitating antibody resembled that of sera from Southeast Asia, with an abrupt increase in incidence at HA titers >160. Within three months after arrival, 50% of the monkeys with HA titers $\cdot$ 80 became anti-

body-negative. Monkeys with higher titers showed essentially no change and a few that were held for one year maintained their original titers. Precipitating antibody was IgG globulin, as was HA antibody in sera with HA titers ≧320. There was suggestive evidence that HA activity in sera with low titers was in the IgM fraction. An occasional serum with a negative HA reaction had precipitating antibody and combining activity; unidentified inhibitors in the serum appeared to be responsible because HA activity could be demonstrated after storage of the same sample for 2–4 weeks at 4 C .

Response of monkeys to challenge with SEB. Monkeys with naturally acquired antibody showed a significant degree of resistance to iv challenge (table 2) but none to alimentary challenge (table 3). Precipitating antibodies were associated with resistance to challenge with 100 ED_{50} iv but were not required for protection against lower doses. Monkeys lacking precipitating antibody, but with HA titers ≧160, almost invariably were resistant to the lower doses. There was no relationship between titers of antibody and resistance to alimentary challenge; of five monkeys that showed no toxic symptoms after challenge with doses >1 ED_{50}, two had no prechallenge antibodies, and the other three had no precipitins.

Signs of illness and their duration differed somewhat for the two routes of challenge. Time of onset following gi exposure was highly variable and was unrelated to dose or antibody level. The first signs of illness appeared in 1.8–9.8 hr with an overall mean time of 3.6 hr (SD, 2.3 hr). Manifestations of illness increased somewhat with size of dose used for challenge, but they were less severe when onset was delayed. Emesis was the primary sign, mild diarrhea accompanied emesis in 20% of the monkeys. Recovery was rapid, usually within 24 hr, and fatalities never occurred. In ancillary studies, challenge with 2,500 ED_{50} gi never resulted in death. In contrast, diarrhea was a prominent symptom after iv challenge, and some monkeys had severe diarrhea without overt vomiting. In monkeys that had no antibody, diarrhea persisted for 2–3 days, and an occasional death occurred after challenge with 100 ED_{50} iv. In animals with antibody, symptoms were milder and of shorter duration. When groups of 4–6 monkeys were challenged with 10–100 ED_{50} by

Table 2. Activity of antibody in monkeys before challenge and illness-response ratio (number ill/number injected) after iv challenge with staphylococcal enterotoxin B (SEB).

Activity of antibody before challenge			Illness-response ratio [challenge dose (ED_{50})]			
HA	AGP	ECA	1	5	25	100
Negative	Negative	<0.1	21/42	31/35	44/46	9/10
10–80	Negative	<0.1–1.0	1/2	4/6	9/15*	10/14
160–10,000	Negative	0.5–2.5	0/3	1/8*	0/8*	3/6
Negative	2	2.5	0/2	...	...	0/1
160–5,120	1–8	2.5–5.0	...	...	0/4*	0/5*

NOTE.—HA = range of titers of HA antibody; AGP = range of titers of precipitating antibody (by agar gel technique); ECA = range of SEB combining activities, μg SEB/ml serum at equivalence.

* $P < .001$, chi square test: comparison with corresponding antibody-negative group.

Table 3. Activity of antibody before challenge of monkeys and illness-response ratio (number ill/number fed) after challenge via alimentary route with staphylococcal enterotoxin B (SEB).

Antibody before conditioning	Activity of antibody before challenge			Illness-response ratio [challenge dose (ED_{50})]		
	HA	AGP	ECA	1	10	100
Negative	Negative	Negative	<0.1	3/6	5/6	...
Positive	Negative	Negative	<0.1	7/8	17/18	9/9
	20–1,280	Negative	<0.1–2.5	1/1	5/8	7/7
	80–1,280	1–4	1.0–2.5	0/1	4/4	3/3

NOTE. HA = range of reciprocal titers of HA antibody; AGP = range of reciprocal titers of precipitating antibody (by agar gel test); ECA = range of SEB combining activities, μg SEB/ml serum at equivalence.

intradermal, subcutaneous, or aerosol routes, the toxic response was similar to that observed after iv challenge. and naturally acquired antibody appeared to be associated with resistance.

Only 25% of 131 monkeys that originally had no HA antibody developed antibody within 4–6 weeks after challenge. The percentage of conversion of sera to HA-positive was the same at all doses by either iv or gi challenge and was unrelated to severity of illness. Magnitude of HA titer after challenge appeared to be related to total dose of SEB administered, regardless of route; following challenge with <10 μg SEB/kg body weight, 22 monkeys that developed HA antibody had a mean titer of 19 (SD, 2), whereas 12 that responded to higher doses had a mean titer of 85 (SD, 11). If monkeys had no preexisting antibody and showed no precipitin response, HA activity appeared only in the IgM fraction of serum. Conversion to a precipitin-positive reaction was limited almost exclusively to monkeys with preexisting HA titers; 39 of 55 monkeys with prechallenge hemagglutinins became precipitin positive in contrast to only 2 of 131 that had negative titers before challenge. Antibody responses of all monkeys that developed precipitins were typical anamnestic reactions, and only IgG globulins were involved. Like the HA response, development of postchallenge precipitins was independent of route of challenge or severity of illness. Prechallenge titer was the primary factor affecting the magnitude of the anamnestic reaction; monkeys with low prechallenge titers showed the greatest response. For groups with the same prechallenge titers, however, response increased with challenge dose. Following gi exposure, monkeys achieved maximal levels of antibody within two weeks, whereas after iv challenge only monkeys with preexisting antibody reached maximal response within two weeks, and other monkeys required 4–6 weeks.

A group of 42 antibody-negative monkeys that recovered from illness induced by 1–25 ED_{50} iv developed no detectable antibody within 10 weeks after exposure. When rechallenged by the same route with 4–25 times the original dose, approximately 40% of the monkeys showed no signs of illness (table 4). The incidence of HA and precipitin responses following reexposure was unrelated to illness and was significantly higher ($P < .001$) than that observed after primary challenge. Temporal course and magnitude of titers of antibody were characteristic of anamnestic reactions. Essentially the same findings were obtained by reexposure of 16 antibody-negative monkeys that resisted the original iv challenge. In contrast to these findings, 42 antibody-negative monkeys that were rechallenged after 10 weeks with 1–100 ED_{50} gi showed no resistance to SEB. Stimulation of antibody response occurred only in monkeys that were fed repeatedly with 100 ED_{50} gi.

Discussion

With the availability of highly purified preparations of staphylococcal enterotoxin B, it became feasible to conduct surveys for, and analysis of, naturally acquired, specific antibody. Evaluation of several serologic techniques indicated that passive hemagglutination of ovine erythrocytes coupled to SEB with bis-diazotized benzidine was the most sensitive method for detecting specific antibody, but that immune precipitation in agar gel provided additional information for characterizing the quality of antibody. Precipitating antibody was always associated with IgG globulins, whereas HA activity was found in all classes of immunoglobulin.

Table 4. Response of recovered, antibody-negative monkeys to iv rechallenge with staphylococcal enterotoxin B (SEB).

Prior iv dose (ED_{50})	Reexposure dose (ED_{50})	No. of monkeys	No. of reactors: Overt illness	No. of reactors: Antibody after challenge* HA	No. of reactors: Antibody after challenge* AGP	Geometric mean titer of responders* HA	Geometric mean titer of responders* AGP
1	25	8	6	7	2	290	1
5	25	17	12	12	3	525	2
25	100	17	7	17	9	770	3

* HA = hemagglutination of red blood cells coupled to SEB with bis-diazotized benzidine; AGP = precipitation in agar gel.

Studies with monkeys revealed that incidence, titer, and type of antibody could be related to known prior experience. Monkeys that lacked prechallenge antibody showed poor serologic response after a single exposure to toxin; 25% developed hemagglutinins of low titer. Repeated exposure was required to produce a high incidence of antibody response and significant titers. Precipitins appeared only after repeated exposures or in monkeys that had antibody before challenge.

Samples of serum obtained from residents of the United States and Southeast Asia showed a high incidence of naturally acquired antibody that in quality and titer appeared to be consistent for individuals within the same geographic group. Titers of antibody were fairly stable; sera with low titers of HA antibody occasionally had precipitating antibody. On repeated sampling, some individuals became antibody-positive without any history of exposure to enterotoxin; similar conversions occurred in monkeys that showed no overt illness after reexposure by parenteral route.

Studies with monkeys indicated that a significant amount of experience with SEB was required to achieve the incidence and quality of natural antibody found in human sera. Reactions of sera from children suggested that this experience was often acquired in preschool years. The source of the antigenic stimulus is unknown, but rechallenge studies with monkeys indicated that parenteral exposure was considerably more effective than alimentary exposure for stimulating production of antibody. With regard to the environmental potential for exposure, Casman [12] reported that only 15% of staphylococcal strains from clinical specimens and fewer than 5% from normal human flora or food samples were capable of producing type B enterotoxin. The high incidence of antibodies to type B in human sera suggests that these estimates may be somewhat low.

Further experimental work will be required to resolve the role of antibody in protection. Our studies indicate that humoral antibody can serve as an index of resistance to parenteral exposure but not to alimentary exposure. Differences in mode of transport of SEB to the site(s) of toxic action may be one of the factors involved. After iv inoculation, and probably after other types of parenteral challenge, transport of toxin is via the vascular system. In the presence of humoral antibody, formation of toxin-antitoxin complexes could neutralize toxic activity either by blocking the normal mechanism of clearance [3, 13] or by steric hindrance of the toxic site on the molecule. In either event, titers of antibody would serve as an index of resistance. Although there are no data available on the fate of toxin following oral challenge, Bergdoll [2] reported that parenteral administration of antiserum could protect against oral challenge, indicating interaction of toxin and antitoxin outside the intestinal lumen. The discrepancy between the quantities required for an iv ED_{50} and for an oral ED_{50} leads to speculation that toxin may be degraded or destroyed in the alimentary tract or in passage across the intestinal barrier. If degradation occurs, haptenic fragments might inhibit neutralization of the toxic moiety by antibody, or if toxin is released slowly into the vascular system, a Danysz-like phenomenon might be operative. In either situation, high levels of antibody would be required for effective neutralization. There is little evidence that secretory antibody localized in the alimentary tract is of primary importance for protection against ingested toxin. Procedures that would be expected to favor production of secretory antibody, such as oral administration of toxin or toxoid, are less effective than parenteral injection in evoking protection against oral challenge [2].

References

1. Bergdoll, M. S., Surgalla, M. J., Dack, G. M. Staphylococcal enterotoxin. Identification of a specific precipitating antibody with enterotoxin-neutralizing property. J. Immun. 83:334–338, 1959.
2. Bergdoll, M. S. Immunization of rhesus monkeys with enterotoxoid B. J. Infect. Dis. 116:191–196, 1966.
3. Rapoport, M. I., Hodoval, L. F., Grogan, E. W., McGann, V., Beisel, W. R. The influence of specific antibody on the disappearance of staphylococcal enterotoxin B from blood. J. Clin. Invest. 45:1365–1372, 1966.
4. Silverman, S. J., Espeseth, D. A., Schantz, E. J. Effect of formaldehyde on the immunochemical and biological activity of staphylococcal enterotoxin B. J. Bact. 98:437–442, 1969.
5. Felsenfeld, O., Nasuniya, N. Staphylococcal antitoxin values in the sera of permanent residents and visitors in Thailand. J. Trop. Med. Hyg. 67:300–303, 1964.
6. Schantz, E. J., Roessler, W. G., Wagman, J., Spero, L., Dunnery, D. A., Bergdoll, M. S. Puri-

fication of staphylococcal enterotoxin B. Biochemistry (Wash.) 4:1011–1016, 1965.

7. Oudin, J. Section III. Immunochemical methods for determining homogeneity of proteins and polysaccharides. Techniques and analysis of the quantitative precipitin reaction. B. Specific precipitation in gels and its application to immunochemical analysis. Meth. Med. Res. 5:335–378, 1952.
8. Boyden, S. V. The adsorption of proteins on erythrocytes treated with tannic acid and subsequent hemagglutination by antiprotein sera. J. Exp. Med. 93:107–120, 1951.
9. Gordon, J., Rose, B., Sehon, A. H. Detection of "non-precipitating" antibodies in sera of individuals allergic to ragweed pollen by an *in vitro* method. J. Exp. Med. 108:38–51, 1958.
10. Wright, S. T. C. A quantitative serum-agar technique. Nature (London) 183:1282–1283, 1959.
11. Scheidegger, J. J. Une micro-méthode de l'immuno-électrophorèse. Int. Arch. Allerg. 7:103–110, 1955.
12. Casman, E. P. Staphylococcal enterotoxin. Ann. N.Y. Acad. Sci. 128:124–131, 1965.
13. Normann, S. J. Why antibody inhibits vascular clearance of staphylococcal enterotoxin B. J. Immun. 104:673–678, 1970.